Research Article

Molecular Cancer Therapeutics

Na,K-ATPase Subunits as Markers for Epithelial-Mesenchymal Transition in Cancer and Fibrosis

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Abstract

Epithelial-to-mesenchymal transition (EMT) is an important developmental process, participates in tissue repair, and occurs during pathologic processes of tumor invasiveness, metastasis, and tissue fibrosis. The molecular mechanisms leading to EMT are poorly understood. Although it is well documented that transforming growth factor (TGF)-β plays a central role in the induction of EMT, the targets of TGF-β signaling are poorly defined. We have shown earlier that Na,K-ATPase β_1-subunit levels are highly reduced in poorly differentiated kidney carcinoma cells in culture and in patients' tumor samples. In this study, we provide evidence that Na,K-ATPase is a new target of TGF-β_1–mediated EMT in renal epithelial cells, a model system used in studies of both cancer progression and fibrosis. We show that following treatment with TGF-β_1, the surface expression of the β_1-subunit of Na,K-ATPase is reduced, before well-characterized EMT markers, and is associated with the acquisition of a mesenchymal phenotype. RNAi-mediated knockdown confirmed the specific involvement of the Na,K-ATPase β_1-subunit in the loss of the epithelial phenotype and exogenous overexpression of the Na,K-ATPase β_1-subunit attenuated TGF-β_1–mediated EMT. We further show that both Na,K-ATPase α- and β-subunit levels are highly reduced in renal fibrotic tissues. These findings reveal for the first time that Na,K-ATPase is a target of TGF-β_1–mediated EMT and is associated with the progression of EMT in cancer and fibrosis. *Mol Cancer Ther; 9(6); 1515–24. ©2010 AACR.*

Introduction

Epithelial-to-mesenchymal transition (EMT) is a process in which polarized epithelial cells undergo multiple biochemical changes to assume a mesenchymal phenotype (1). In general, epithelia form an effective barrier between underlying tissue and external media. To perform this function, epithelial cells possess extensive junctional networks that physically separate the plasma membrane into apical and basolateral domains. The junctional network also promotes adhesion, facilitates intercellular communication, restricts motility, and thus preserves tissue integrity and permits epithelial cells to function as a cohesive unit (2). The phenotype of epithelial cells with apical-basal polarity and junctional complexes is referred to as well-differentiated phenotype. Carcinoma arising from epithelial tissue is by far the most prevalent form of cancer accounting for ~90% of human malignancies. During carcinoma progression to advanced disease, epithelial cells often lose their well-differentiated phenotype and adopt a fibroblast-like, mesenchymal phenotype in a sequence of events often called EMT. This process involves the loss of cell-cell adhesion and intercellular junctions, destruction of the basement membrane, reorganization of the actin cytoskeleton, enhanced migratory capacity and invasiveness, activation of transcription factors, and downregulation of epithelial markers and expression of mesenchymal genes (3). Molecular and morphologic features of EMT often correlate with poor histologic differentiation and destruction of tissue integrity, and, in general, are associated with advancement in disease and deterioration in tissue function (4). Although it is recognized that EMT is an important event during cancer progression as well as in fibrotic disease, molecular mechanisms leading to EMT and target molecules affected during this process are poorly understood.

A key molecule implicated in EMT is transforming growth factor-β_1 (TGF-β_1; refs. 5, 6). The TGF-β growth factor superfamily comprises TGF-βs, bone

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morphogenetic proteins (BMP), activins, and other related proteins. The functional complex of TGF-β family receptors at the cell surface consists of two type II and two type I transmembrane serine/threonine receptors (5, 6), which exist as homodimers in the absence of ligand. Ligand binding to the type II receptor recruits the type I receptor into a heteromeric complex, resulting in the transphosphorylation of type I receptor by type II receptor. Following phosphorylation of Smad2 or Smad3 by the activated type I receptor, a heteromeric complex is formed with Smad4, resulting in the translocation of the complex to the nucleus to directly or indirectly regulate gene transcription. In addition to Smad-dependent pathways, TGF-β also activates Smad-independent pathways such as the Erk, c-Jun-NH_2-kinase, and p38 mitogen-activated protein kinase (MAPK) signaling pathways (6, 7). The mechanism by which TGF-β_1–mediated signaling in epithelial cells triggers EMT and its consequences remains to be clarified.

Na,K-ATPase is an abundantly expressed protein in epithelial cells and plays a crucial role in kidney function. Localized to the basolateral plasma membrane, the oligomeric Na,K-ATPase catalyzes an ATP-dependent transport of three Na^+ out and two K^+ into the cell per pump cycle to maintain Na^+ and K^+ gradients across the plasma membrane. This Na^+ and K^+ homeostasis is necessary to regulate the functions of the various ion and solute transporters in epithelial cells. Na,K-ATPase is composed of two essential polypeptide subunits, the α-subunit (~112 kDa; ref. 8) and the β-subunit (~55 kDa; ref. 9), and an optional regulatory γ-subunit with tissue-specific expression (~7 kDa; ref. 10). Of the four α- and three β-subunit isoforms known, α_1 (NaK-α_1) and β_1 (NaK-β_1) are predominantly expressed in kidney (11).

We have shown that in addition to its epithelial transport function, Na,K-ATPase plays a fundamental role in the formation and maintenance of a well-differentiated, polarized epithelial phenotype in mammalian cells (12–16). Inhibition of Na,K-ATPase activity prevents the polarization of epithelial cells and disrupts tight junction structure and function in polarized epithelial monolayers (13, 15). Recently, we and others have shown that the Na,K-ATPase β_1-subunit (NaK-β_1) functions as a cell-cell adhesion molecule (16–19), is localized to the apical junctional complex in polarized epithelial cells (13), and may also function as a tumor suppressor (20). Na,K-ATPase also regulates tight junction function and formation during mouse preimplantation and blastocyst development (21, 22). In *Drosophila*, homologues of both NaK-α_1 and NaK-β_1 are localized to septate junctions (which are functionally similar to tight junctions in epithelial cells) and are involved in regulating their permeability (23, 24). Subsequent studies showed that the *Drosophila* Na,K-ATPase has an ion pump–independent role in junction formation (25) and that Na,K-ATPase belongs to a new group of polarity proteins (26). In zebrafish, Na,K-ATPase is essential for epithelial integrity during heart morphogenesis (27) and lumen formation in the gut (28). Together, these studies indicate that Na,K-ATPase has a conserved role in the regulation of the well-differentiated phenotype of epithelial cells (29).

In this study, using LLC-PK1 cells, a well-differentiated kidney epithelial cell line that undergoes TGF-β–mediated EMT (30), we show that Na,K-ATPase is a target molecule of the TGF-β_1 signaling pathways. We provide evidence that reduced Na,K-ATPase expression and function are accompanied by increased intracellular sodium levels and activation of MAPK signaling, and might be one of the events associated with the loss of the polarized epithelial phenotype during TGF-β_1–induced EMT in kidney epithelial cells.

Materials and Methods

Cell culture, small interfering RNA, and transfections

LLC-PK1 cells (American Type Culture Collection) were maintained in DMEM with 10% fetal bovine serum, 2 mmol/L L-glutamine, 25 U/mL penicillin, and 25 μg/mL streptomycin. TGF-β_1 (R&D) was added to subconfluent cultures at 4 ng/mL in serum-free DMEM for the time indicated after serum starvation for 4 to 6 hours. Control cells were serum starved in parallel.

Small interfering RNA (siRNA) against NaK-β_1 (5′-AAAAGUGAUGCUGCUCACCAUCA-3′) and a scrambled control oligoribonucleotide (5′-AACUGCCGUACUAAGUUAAGACA-3′) were from Dharmacon. The uniqueness of the sequences was confirmed using the Genbank/EBI database. Cells were transfected with 150 nmol/L NaK-β_1 or control oligonucleotide using Cytofectene (Bio-Rad) and harvested 72 hours post-transfection. For the stable knockdown of NaK-β_1 in Caki-1 cells, the NaK-β_1 siRNA sequence was cloned into pSilencer 5.1 (Ambion). Caki-1 cells (American Type Culture Collection) were transfected with the cDNA using the calcium phosphate method, and stable clones of NaK-β_1 knockdown Caki-1 cells were selected with 10 μg/mL puromycin.

Flag-tagged Smad7 cDNA was kindly provided by Dr. H.L. Moses (Vanderbilt University, Nashville, TN). Transfections were done with the FuGene6 reagent (Roche).

Antibodies

Antibodies against ZO-1 and occludin were obtained from Invitrogen Corp.; antibodies against E-cadherin were from BD Biosciences; Flag-epitope (DYKDDDK), p44/42-MAPK (Erk1/2), and phospho-p44/42-MAPK (Erk1/2) antibodies were from Cell Signaling; TGF-β RII antibodies were from Abcam, Inc.; and fibronectin, β-actin, and smooth muscle actin (SMA) antibodies were from Sigma Co. Antibodies against NaK-α_1 (M7-PB-E9) and NaK-β_1 (M17-P5-F11) were kindly provided by Dr. William Ball, Jr. (University of Cincinnati, Cincinnati, OH). Horseradish peroxidase–conjugated secondary

antibodies were from Cell Signaling; FITC- or CY3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories.

Immunoblotting

Cell lysates for NaK-α_1, NaK-β_1, fibronectin, SMA, and E-cadherin were prepared as described earlier (13, 16) in a buffer containing 95 mmol/L NaCl, 25 mmol/L Tris (pH 7.4), 0.5 mmol/L EDTA, 2% SDS and protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL each of antipain, leupeptin, and pepstatin), or in a buffer of 20 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium glycerolphosphate, 1 mmol/L sodium orthovanadate, and protease inhibitors (phospho-Erk1/2, Erk1/2). Equal amounts of protein were separated by SDS-PAGE and immunoblotted as described (13, 16).

Immunofluorescence

Immunofluorescence was done as previously described (15, 16, 31). Epifluorescence pictures were captured with a SPOT CCD camera and the SPOT imaging software, version 4.0.4 (Diagnostic Instruments, Inc.) attached to an Olympus AX70 microscope.

TGF-β–responsive promoter assay

LLC-PK1 cells transiently transfected with SBE-luc (Smad4 Binding Element luciferase) alone, Smad7-Flag

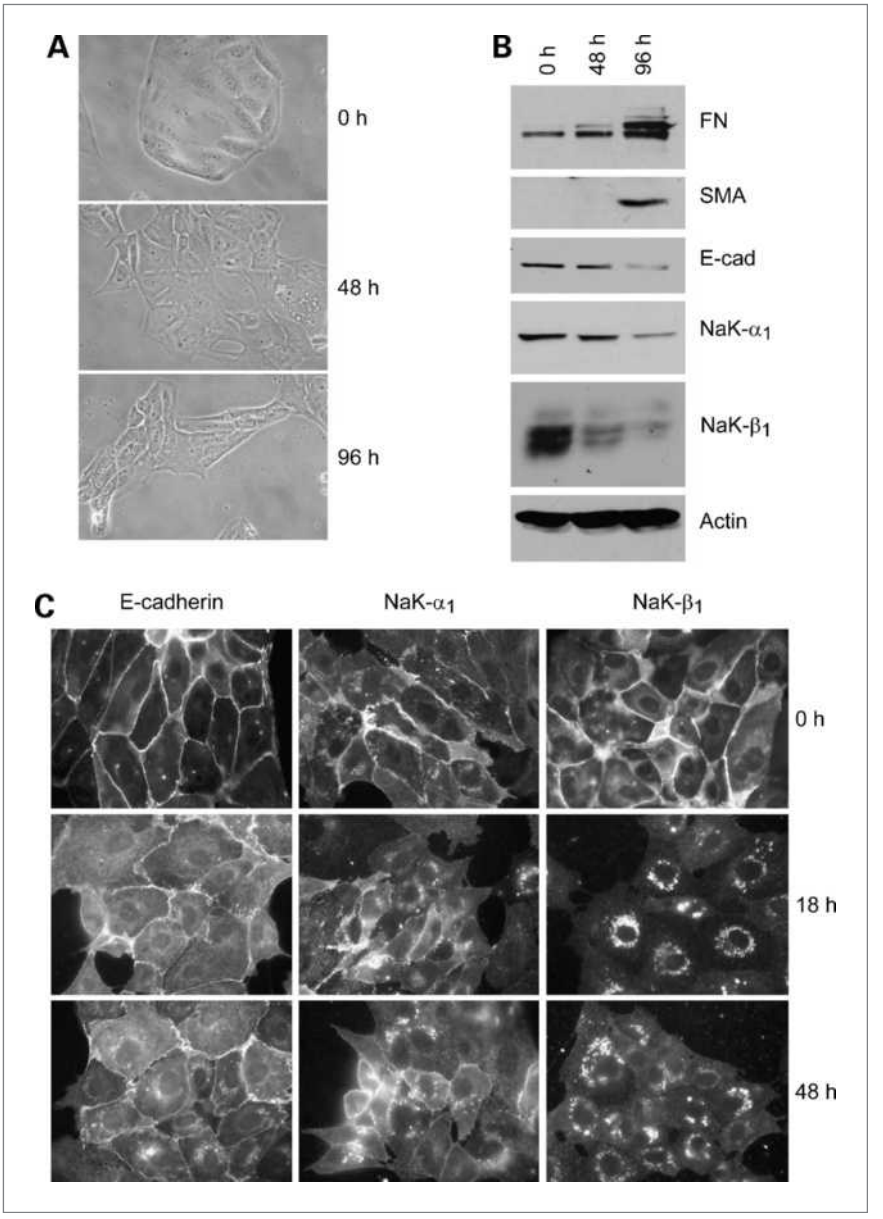

Figure 1. LLC-PK1 cells undergo TGF-β_1–mediated EMT. A, phase-contrast microscopy of control (0 h) and TGF-β_1–treated (48 and 96 h) cells. B, representative immunoblots for EMT markers fibronectin (FN) and α-SMA (SMA), E-cadherin (E-cad), Na,K-ATPase α-subunit (NaK-α_1), and β-subunit (NaK-β_1). Actin immunoblot confirms equal loading. C, immunostaining for E-cadherin, NaK-β_1, and NaK-α_1. NaK-β_1 redistributed from the plasma membrane in control cells to intracellular vesicles in TGF-β_1–treated cells as early as 18 hours after TGF-β_1 treatment.

alone, or SBE-luc and Smad7-FLAG were treated with TGF-β_1 48 hours after transfection. Luciferase activity was measured after 24 hours of TGF-β_1 treatment using the Dual-Luciferase Reporter Assay System (Promega) as described (32). The firefly luciferase activity was normalized to the activity of the internal control (*Renilla* luciferase, pRL vector) that was cotransfected along with the reporter vector.

Real-time quantitative PCR

Primer sets used were as follows: NaK-β_1 forward, 5′-TTACCCTTACTACGGCAAGCTCCT-3′, reverse, 5′-TTCAGTGTCCATGGTGAGGTTGGT-3′; *Snail* forward, 5′-*TCACCGGCTCCTTCGTCCTTC*-3′; reverse, 5′-*TCCTTGTTGCAGTATTTGCAGTT*-3′; glyceraldehyde-3-phosphate dehydrogenase forward, 5′-GTGAAGGTCG-GAGTCAACGG-3′; reverse, 5′-TGATGACAAGCTTC-CCGTTCTC-3′. Reactions were run in triplicate on an iCycler iQ with iQ SYBR Green supermix detection (both Bio-Rad). Variation in cDNA loading was normalized to glyceraldehyde-3-phosphate dehydrogenase simultaneously amplified in each well.

Rubidium transport assay and atomic emission spectroscopy

Na,K-ATPase activity was determined as the ouabain-sensitive $^{86}Rb^+$ transport as described (16). The samples were normalized to the protein content.

Intracellular ion concentrations were measured with an inductively coupled plasma atomic emission spectrometer (Vista Axial 730; Varian) as described (16). The concentrations for Na^+ (588.995 nm), K^+ (766.941 nm), and Mg^{2+} (285.213 nm) were determined and Na^+ and K^+ concentrations were normalized to the total Mg^{2+} content (internal control).

Cell volume measurements

The procedure described by Kletzien and colleagues (33) was modified for measuring the cell volume of polarized epithelia. Cells grown on 25-mm Millipore Isopore, 0.8-μm pore membranes coated with bovine tendon collagen previously cross-linked in ammonia vapor were rinsed and incubated in glucose-free MOPS–buffered saline (pH 7.35), 290 mOsm, containing 0.1 mmol/L ^{14}C-3-O-methyl-D-glucose (DuPont, NEN) at an activity of 0.1 μCi/mL. After 50 minutes of incubation, filters were rinsed in ice-cold saline containing 0.1 mmol/L phloretin and lysed with 0.4 mol/L perchloric acid. After centrifugation, supernatant samples were taken for liquid scintillation counting and precipitates were analyzed for DNA by the diphenylamine spectrophotometric procedure. Using steady-state conditions as above allows for conversion of micromoles of 3-O-methyl-D-glucose per microgram DNA to milliliters of intracellular water per microgram DNA.

Immunohistochemistry

Following Institutional Review Board approval, sections from biopsies were obtained from normal kidney and from three different diabetic patients diagnosed with mild, moderate, or severe diabetic nephropathy. NaK-α_1 and NaK-β_1 were detected with mouse monoclonal antibodies M7-PB-E9 and M17-P5-F11, respectively, which have been used for the immunohistochemical staining of human kidney previously (11). Standard VECTASTAIN ABC immunohistochemical staining was used (Vector Laboratories) and diaminobenzidine was used as the chromogen. The sections were counterstained with Gill's Hematoxylin plus bluing reagent.

Normal sections were obtained from kidneys of noninduced control rats and diabetic kidney sections from streptozotocin-induced rats (12 wk). NaK-α_1 and NaK-β_1 were detected with the NaK-α_1 (M7-PB-E9) antibody and a polyclonal antibody against NaK-β_1 (generous gift of Dr. William Ball, University of Cincinnati, Cincinnati, OH) that both recognize rat Na,K-ATPase subunits. The sections were counterstained with Gill's Hematoxylin. For all stainings, complementary slides processed in the same manner minus primary antibody served as negative controls.

Statistics

Results are expressed as means ± SEM. The statistical significance of differences was calculated using Student's *t* test.

Results

Na,K-ATPase is a target of TGF-β_1 signaling in proximal tubule kidney epithelial cells

LLC-PK1 cells, a porcine proximal tubule-like kidney cell line, are well established as a cell culture model of TGF-β_1–induced EMT (30). Consistent with previous studies, treatment of LLC-PK1 cells with TGF-β_1 induced a fibroblastic phenotype (Fig. 1A) with expression of fibronectin and α-SMA, and reduced levels of the cell adhesion molecule E-cadherin (Fig. 1B). In addition, TGF-β_1–induced EMT was accompanied by drastically reduced NaK-β_1 (48 h: 48 ± 15%; 96 h: 27 ± 10% of control) and NaK-α_1 (48 h: 81 ± 12%; 96 h: 32 ± 11% of control) protein levels.

As expected, immunofluorescence analysis showed NaK-β_1 and NaK-α_1 predominantly localized to sites of cell-cell contact at the plasma membrane in control cells. In contrast, these subunits were localized to distinct intracellular vesicles surrounding the nucleus in TGF-β_1–treated cells (Fig. 1C). More importantly, the redistribution of both Na,K-ATPase subunits from the plasma membrane to intracellular vesicles was observed early on, after 18 hours of TGF-β_1 treatment, suggesting that altered Na,K-ATPase localization is an early event in the TGF-β_1–mediated induction of EMT. Consistent with the intracellular localization of the Na,K-ATPase subunits, the regulation of the NaK-β_1 protein level seemed to occur at the posttranslational level because quantitative real-time PCR did not show significant differences in NaK-β_1 mRNA levels between control and TGF-β_1–treated cells (Supplementary Fig. S1A). We have

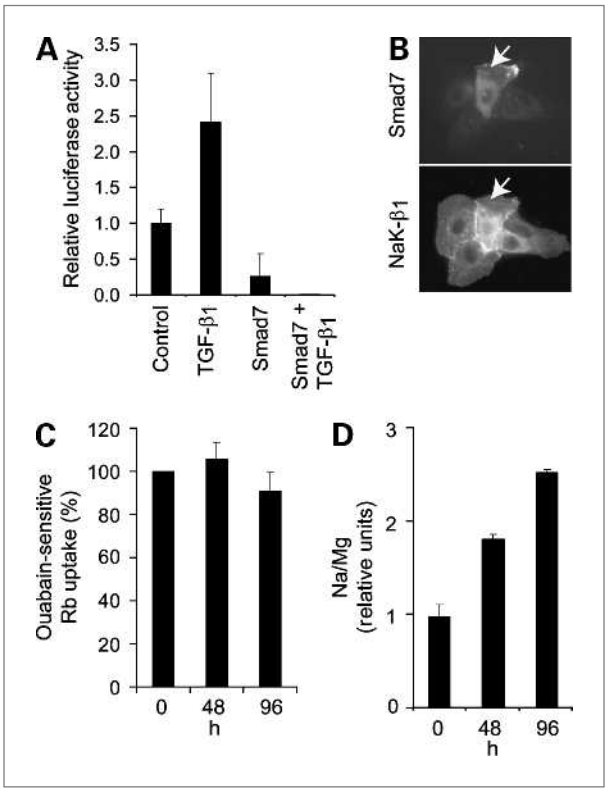

Figure 2. Smad dependence and effects of altered Na,K-ATPase in TGF-β_1–treated cells. A, TGF-β–responsive SBE-Luc reporter activity. LLC-PK1 cells transiently transfected with SBE-luc alone (control, TGF-β_1) or SBE luc and Smad7-FLAG (Smad 7, Smad7 + TGF-β_1), and treated with TGF-β_1 for 24 hours (TGF-β_1, Smad7 + TGF-β_1) or vehicle (control, Smad7). The ratio of the Firefly luciferase activity over the *Renilla* luciferase is expressed as relative activity compared with control cells. Columns, mean from three independent determinations; bars, SD. B, transiently expressed SMAD7 in TGF-β_1–treated cells (72 h) was detected with anti-Flag antibody. Note the intense staining of NaK-β_1 on the plasma membrane in Smad7-expressing cells (arrow). Representative image from two independent transfections is shown. C, Na,K-ATPase activity in TGF-β_1–treated cells. Ouabain-sensitive $^{86}Rb^+$ uptake in control (0 h) and TGF-β_1–treated cells (48 and 96 h) was determined as described in Materials and Methods. D, TGF-β_1 treatment results in increased intracellular sodium. $[Na^+]_i$ in control and TGF-β_1–treated cells was determined by atomic emission spectrometry. Columns, mean from four (C) or two (D) independent determinations done in triplicates; bars, SD.

previously shown that the transcription factor Snail represses NaK-β_1 in cancer cells (34). We did not find any significant differences in Snail mRNA levels in control and TGF-β_1–treated cells (Supplementary Fig. 1B), further supporting that reduced NaK-β_1 levels in TGF-β_1–treated cells are due to posttranslational regulation.

To test whether the relocation of NaK-β_1 from the plasma membrane is a Smad-dependent or Smad-independent process, we transiently expressed Smad7 in LLC-PK1 cells. Smad7 inhibits TGF-β–dependent signaling by preventing Smad 4 from entering the nucleus following TGF-β_1 treatment and has been reported to inhibit Smad-dependent TGF-β1 signaling in other kidney epithelial cells (35, 36). Similarly, TGF-β_1–responsive promoter activity as assessed by SMAD binding element activity was inhibited in LLC-PK1 cells transiently expressing Smad7 (Fig. 2A). Immunofluoresence of Smad7-expressing LLC-PK1 cells treated with TGF-β_1 revealed that NaK-β_1 still localized to the plasma membrane (Fig. 2B, arrow), whereas untransfected cells showed the previously observed vesicular staining pattern confirming that NaK-β_1 expression and distribution are targets of Smad-dependent TGF-β_1 signaling.

TGF-β_1–induced EMT is accompanied by increased intracellular sodium

Earlier, we observed that both Na,K-ATPase subunit levels (16) and enzyme activity (13–15) are essential for a polarized epithelial phenotype. Although we did not find a significant difference in Na,K-ATPase activity between control and TGF-β_1–treated cells in ouabain-sensitive $^{86}Rb^+$ uptake experiments (Fig. 2C) or in total cellular $^{86}Rb^+$ uptake (data not shown), atomic emission spectrometry studies revealed increased intracellular sodium concentrations ($[Na^+]_i$) in TGF-β_1–treated cells with a 1.85- ± 0.05-fold and 2.59- ± 0.03-fold increase in $[Na^+]_i$ after 48 and 96 hours of TGF-β_1 treatment, respectively (Fig. 2D). Intracellular potassium levels were not altered (data not shown). Increased $[Na^+]_i$ seems to be a common feature of cells undergoing EMT. We found that poorly differentiated bladder, colon, and pancreatic cancer cells that express low levels of NaK-β_1 (34) have higher $[Na^+]_i$ levels when compared with well-differentiated cells of similar origin (Supplementary Fig. S2).

Consistent with the increase in $[Na^+]_i$, the water content of TGF-β_1–treated cells was 1.36-fold higher ($P < 0.001$) compared with control cells. Although these data seem contradictory at first, it is well known that high $[Na^+]_i$ will drive the Na,K-ATPase activity, resulting in a higher turnover rate per molecule, which is similar to our earlier findings in murine sarcoma virus–transformed Madin-Darby canine kidney cells. Murine sarcoma virus-transformed Madin-Darby canine kidney cells that express low NaK-α_1 and NaK-β_1 levels did not display major differences in $^{86}Rb^+$ uptake, although $[Na^+]_i$ was 2.8-fold higher compared with Madin-Darby canine kidney cells (16). Together, these data show that TGF-β_1–mediated EMT in LLC-PK1 cells is associated with increased intracellular sodium levels and accompanied change in cell volume.

NaK-β_1 expression is essential to maintain a well-differentiated phenotype

Because we observed reduced NaK-β_1 expression during the loss of the epithelial phenotype in TGF-β_1–treated cells, we tested whether specific knockdown of this protein alters the phenotype of LLC-PK1 cells. Indeed, reducing NaK-β_1 protein levels by 63 ± 8% in LLC-PK1 cells using an RNAi oligomer approach (Fig. 3A) resulted in the loss of the epithelial phenotype. Although E-cadherin levels remained unaffected (Fig. 3A), NaK-β_1 siRNA cells displayed a fibroblastic morphology, whereas

the parental cell line and scrambled oligomer–transfected control cells showed the cobblestone-like morphology typical of well-differentiated epithelial cells (Fig. 3B). Because NaK-β_1 is essential for the transport and stability of the NaK-α_1, the reduced NaK-β_1 level was accompanied by a reduced NaK-α_1 level (Fig. 3A), which was also observed in TGF-β_1–treated cells (Fig. 1B). Furthermore, stable knockdown of NaK-β_1 in the human renal clear cell carcinoma cell line, Caki-1 induced a fibroblastic morphology, accompanied by the expression of the EMT markers fibronectin and α-SMA (Supplementary Fig. S3). Thus, the loss of NaK-β_1 expression is sufficient to induce a fibroblastic phenotype.

Using a complementary approach (overexpression of NaK-β_1), we further confirmed that high NaK-β_1 expression levels delay the onset of phenotypic changes and induction of EMT markers. TGF-β_1 treatment of LLC-PK1 cells expressing human NaK-β_1 (LLC-PK1-NaK-β_1; Fig. 3C) showed minimal change in morphology at 48 and 96 hours, whereas control cells showed a typical mesenchymal phenotype (Fig. 3E). Although NaK-β_1 levels were reduced in control and LLC-PK1-NaK-β_1 cells following TGF-β_1 treatment (Fig. 3C), LLC-PK1-NaK-β_1 cells had twice as much of NaK-β_1 compared with control cells at 48 and 96 hours of treatment (Fig. 3C). Fibronectin and SMA levels inversely correlated with NaK-β_1 levels. Fibronectin and α-SMA levels in TGF-β_1–treated LLC-PK1-NaK-β_1 cells were only 53% and 56%, respectively, of the levels of control cells (Fig. 3D). Taken together, these data show that reduction of NaK-β_1 levels beyond a certain threshold is a critical step for the induction of EMT in kidney tubule cells.

NaK-β_1 knockdown activates Erk1/2 signaling

Activation of MAPK signaling is a prerequisite for TGF-β_1–induced EMT (7). Although Smad signaling pathways have been well described, much less is known about the activation of Erk1/2 signaling upon TGF-β_1 treatment. In LLC-PK1 cells, TGF-β_1 induced a transient activation of Erk1/2 that peaked at 10 minutes and diminished after 30 minutes of treatment (Fig. 4A). However, after 48 and 96 hours of TGF-β_1 treatment, Erk1/2 activation was increased when compared with non-treated LLC-PK1 control cells (Fig. 4B). Interestingly,

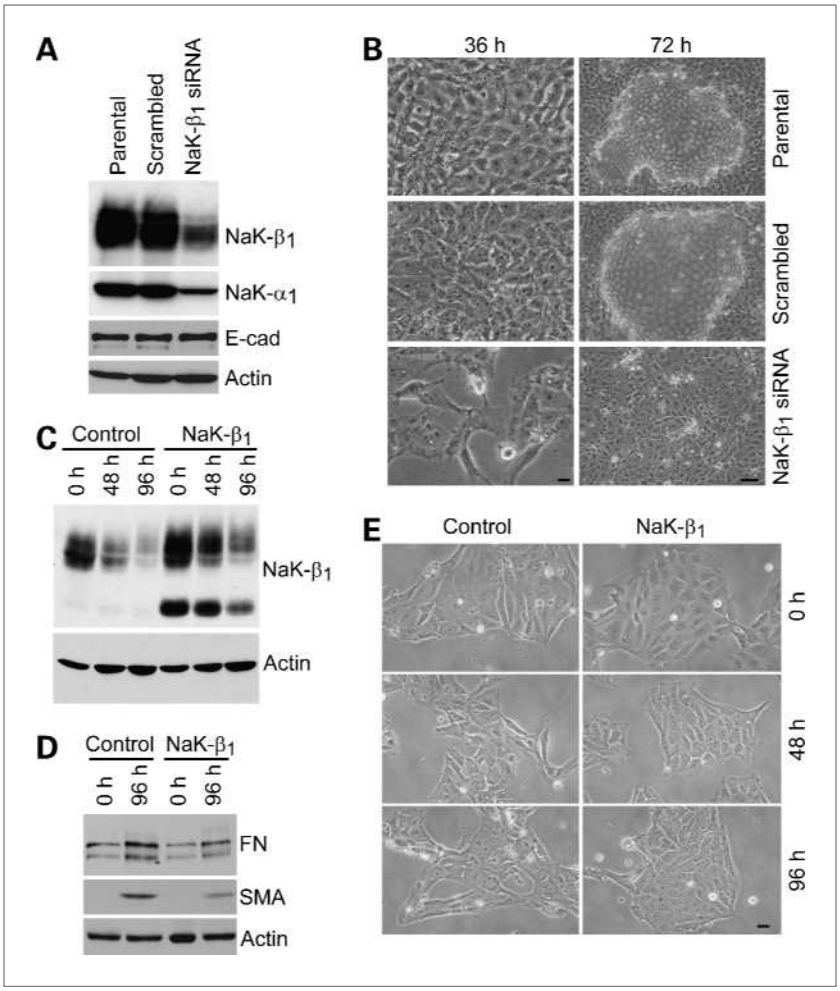

Figure 3. Effect of NaK-β_1 knockdown and overexpression in LLC-PK1 cells. A and B, RNAi-mediated knockdown of NaK-β_1. A, immunoblot analysis for NaK-β_1, NaK-α_1, and E-cadherin in parental cells, control cells transfected with scrambled oligo (scrambled), and NaK-β_1 knockdown cells (NaK-β_1 siRNA). B, NaK-β_1 siRNA cells are fibroblastic (36 h posttransfection) and do not form characteristic cell domes in confluent monolayers (72 h). Bars, 20 μm (36 h); 100 μm (72 h). C to E, ectopic expression of NaK-β_1 delays the onset of TGF-β_1–mediated EMT in LLC-PK1 cells. Immunoblots for NaK-β_1 (C) and EMT markers fibronectin (FN) and α-SMA (SMA; D) and morphology (E) of control and NaK-β_1–expressing cells upon TGF-β_1 treatment. Bar, 20 μm.

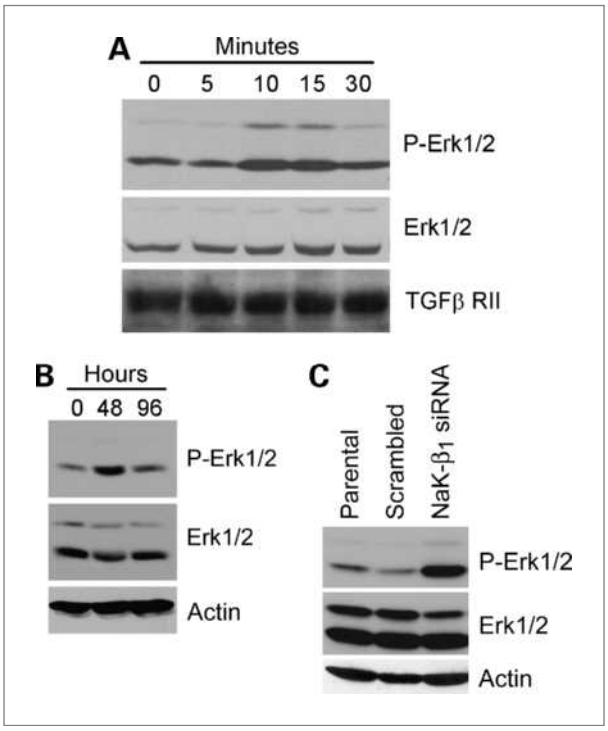

Figure 4. Activation of Erk1/2 signaling in TGF-β_1–treated and NaK-β_1 knockdown cells. Immunoblot analysis for activated and total Erk1/2 in TGF-β_1–treated LLC-PK1 cells immediately after treatment (A) and after long-term treatment (B). Duration of TGF-β_1 treatment is indicated in minutes (A) or hours (B). TGF-β RII (A) and actin (B) immunoblots are included to confirm equal amounts of protein used in analysis. C, activation levels of Erk1/2 in NaK-β_1 siRNA cells and corresponding parental and scrambled oligo control cells. Actin immunoblot confirms equal loading.

sustained activation of Erk1/2 correlated with reduced NaK-β_1 expression (Fig. 1B), suggesting that reduced NaK-β_1 levels might be associated with Erk1/2 activation. Indeed, in NaK-β_1 siRNA cells, activated Erk1/2 levels were 3- and 4-fold higher compared with parental or scrambled oligo–transfected LLC-PK1 cells, respectively (Fig. 4C), suggesting that NaK-β_1 levels are at least in part involved in sustained activation of Erk1/2 in TGF-β_1–mediated EMT.

Na,K-ATPase subunit levels are reduced in different types of EMT

EMT is a process not only associated with cancer progression but also with fibrosis. In fact, LLC-PK1 cells have been used as a model to study the transdifferentiation of epithelial cells into interstitial fibrotic cells observed in fibrotic tissue (30). Because Na,K-ATPase subunits were affected in this cell culture system, we examined the NaK-β_1 and NaK-α_1 levels in well-defined fibrotic tissues by immunohistochemical analysis of kidney tissue sections from diabetic patients. Diabetic nephropathy is the most common contributor to end-stage renal disease and emerging evidence suggests that EMT of renal tubular epithelial cells to myofibroblasts is an important event in renal tubulointerstitial fibrosis (37–39). Renal biopsy samples were obtained from patients diagnosed with mild, moderate, and severe diabetic nephropathy and compared with normal kidney tissue sections. NaK-β_1 and NaK-α_1 were clearly reduced in fibrotic tissues compared with normal tissues (Fig. 5). In addition, the immunohistochemistry for NaK-α_1 and NaK-β_1 on tissue sections obtained from streptozotocin-induced diabetic rats revealed considerably reduced staining intensity in nephropathic tissues compared with normal tissues of noninduced rats (Fig. 6). Thus, reduced Na,K-ATPase subunit expression seems to be associated with fibrotic as well as cancerous EMT.

Discussion

In this study, we provide the first evidence that Na,K-ATPase is a target of TGF-β_1 signaling during the induction of EMT. We show that following the treatment of renal proximal tubule cells with TGF-β_1, NaK-β_1 surface expression is reduced before well-characterized EMT markers, such as E-cadherin, and the induction of fibronectin and α-SMA. We validated the significance of NaK-β_1 in the induction of EMT by two complementary approaches. RNAi-mediated specific knockdown of NaK-β_1 resulted in the loss of the epithelial phenotype, whereas the ectopic expression of NaK-β_1 delayed the induction of a fibroblastic phenotype and reduced the levels of fibronectin and α-SMA following TGF-β_1 treatment. We further show that NaK-β_1 reduction is a common event in EMT irrespective of the type of EMT.

RNAi-mediated specific knockdown of NaK-β_1 is sufficient to induce a fibroblastic phenotype of LLC-PK1 cells. We have shown earlier that NaK-β_1 expression is reduced in a wide variety of poorly differentiated cell lines (34) and that NaK-β_1 repletion induces a well-differentiated phenotype in kidney epithelial cells (16). Collectively, these data show that NaK-β_1 subunit function is important to maintain the well-differentiated phenotype of epithelial cells. We and others have shown that NaK-β_1 functions as a cell-cell adhesion molecule (16–19) and loss of its cell adhesion function upon its reduced plasma membrane expression might be associated with the EMT in TGF-β_1–treated cells. In addition, we have shown earlier that E-cadherin is less stably associated with the actin cytoskeleton when the NaK-β_1 levels are low (16). Because the translocation of NaK-β_1 from the plasma membrane occurs before E-cadherin, we suggest that reduced surface expression of NaK-β_1 might lead to the destabilization of the interaction of E-cadherin with the actin cytoskeleton. The loss of the cell-cell adhesion function of E-cadherin, which is dependent on its stable association with the actin cytoskeleton (40), might then further contribute to the phenotypic changes associated with EMT.

The transcription factor Snail, which suppresses E-cadherin transcription, plays an important role in TGF-β_1–mediated EMT (41, 42). We have shown that

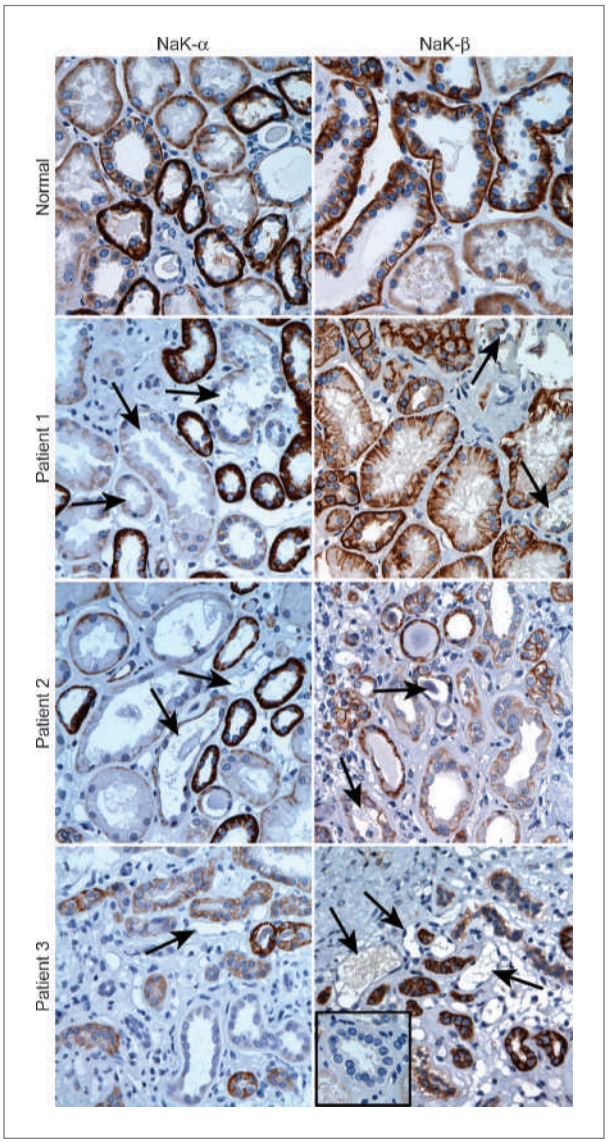

Figure 5. Immunohistochemistry for NaK-α_1 and NaK-β_1 in kidneys of patients diagnosed with mild, moderate, or severe diabetic nephropathy (patients 1, 2, and 3, respectively). NaK-α_1 and NaK-β_1 show intense staining in tubules of normal kidney. Note the fibroblastic phenotype of epithelial cells in tubules undergoing EMT in patients with diabetic nephropathy (arrows). These tubules show no or little NaK-β_1 and NaK-α_1 staining. Insert in patient 3, NaK-β_1 is negative control.

Snail also suppresses NaK-β_1 transcription (34). Interestingly, in TGF-β_1–treated LLC-PK-1 cells, NaK-β_1 mRNA levels were not affected. Our data also indicate that Snail levels were not increased in TGF-β_1–treated cells (Supplementary Fig. S1), suggesting that the reduced NaK-β_1 levels are primarily due to posttranslational mechanisms. This notion is further supported by our observations in LLC-PK1 cells ectopically expressing NaK-β_1. Although NaK-β_1 expression is regulated by a cytomegalovirus promoter in these cells, the protein level was ultimately reduced following TGF-β_1 treatment (Fig. 3C). Further experiments will be necessary to understand the posttranslational mechanisms that lead to reduced NaK-β_1 levels during TGF-β_1–induced EMT in kidney epithelial cells.

In this study, we report for the first time that TGF-β_1–induced EMT is associated with increased $[Na^+]_i$ levels. We consistently observed that TGF-β_1–treated cells appear larger than control cells, a phenomenon also observed by others (43). Increased intracellular sodium could explain such an increase in cell size due to osmotic regulation, a well-documented phenomenon, and is consistent with our observation of increased water content in TGF-β_1–treated cells.

TGF-β_1 treatment activates Erk1/2 transiently during early time points, and after 30, minutes the phospho Erk1/2 level is drastically reduced. At this time point, we did not detect any change in NaK-β_1 levels or localization. Interestingly, the knockdown of NaK-β_1 constitutively activated Erk1/2 in LLC-PK1 cells as well as in other epithelial cell lines (data not shown). We also showed that reduced NaK-β_1 is associated with increased Erk1/2 *in vivo* in tumor xenograft models (20). Thus, we propose that in general, the loss of NaK-β_1 is associated with the activation of Erk1/2. In the context of TGF-β_1–induced EMT, reduced NaK-β_1 might be involved in the sustained activation of Erk1/2 that was observed after 48 and 96 hours of treatment and is involved in events leading to cancer progression.

Recently, three subtypes of EMT have been suggested, which include mesenchymal EMT during gastrulation or

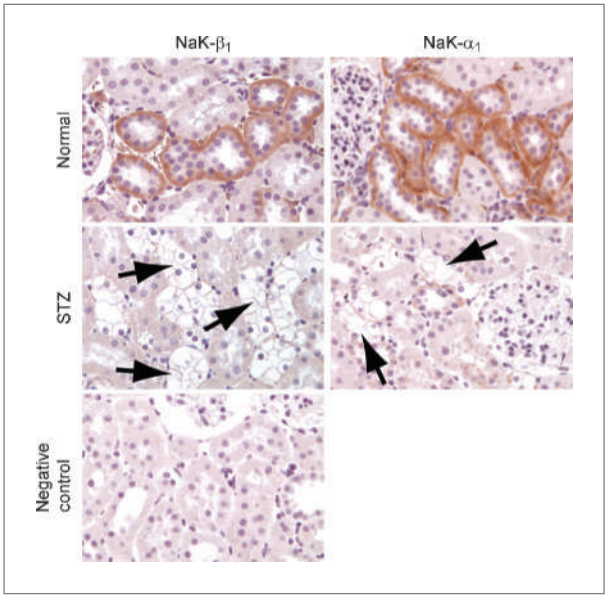

Figure 6. Immunohistochemistry for NaK-α_1 and NaK-β_1 of diabetic kidneys of streptozotocin (STZ)-induced rats. Staining shows reduced NaK-β_1 and NaK-α_1 staining in kidney tubules of streptozotocin-induced rats compared with normal kidney. Arrows, glycogen-granule accumulation in tubular epithelium, a morphologic change often associated with sustained hyperglycemia.

neural crest formation (type 1), EMT in the context of inflammation and fibrosis (type 2), and EMT of cancer with invasion and metastasis (type 3; refs. 1, 3). Loss of Na,K-ATPase expression during EMT in cancer has been reported in numerous studies from our group (11, 16, 20, 44, 45) and others (46–49). We now provide evidence that NaK-β_1 and NaK-α_1 are reduced during EMT in fibrotic tissues in humans as well as in animals. These data are consistent with the idea that Na,K-ATPase is a potential marker for both type 2 and type 3 EMT. Although EMT type 2 and EMT type 3 have in common the loss of the epithelial phenotype, they have distinct pathologic consequences. During fibrosis, epithelial cells transdifferentiate into myofibroblasts that secrete an extensive amount of extracellular matrix. As these cells are embedded in this matrix, tissue architecture and function are lost. On the other hand, EMT during cancer progression results in the increased motility, invasion, and metastatic potential of cancer cells. Type 2 and type 3 EMT are mediated at least in part by the same growth factors and can derive from the same cells (e.g., renal clear cell carcinoma and renal fibrosis are thought to originate from renal proximal tubule cells). Our results indicate that loss of NaK-β_1 is associated with EMT in cancer and fibrosis, and occurs early on (Fig. 1B and C). Thus, the loss of NaK-β_1 expression might have important significance in the pathologic consequences associated with loss of the epithelial phenotype and EMT. However, the molecular factors that contribute to one type of EMT over the other remain to be determined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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